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PRINCIPAL INVESTIGATOR: Doctor Eitan Friedman

CONTRACTING ORGANIZATION: Chaim Sheba Medical Center
Tel Hashomer, 52621 Israel

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Abstract

Stress fractures (SF's) affect a substantial portion of military recruits. We are investigating the hypothesis that a germline mutations in the procollagen genes or specific allelic patterns in other candidate genes confer genetic susceptibility to SF. Clinical, biochemical epidemiological and bone scan data on Israeli male soldiers diagnosed with high grade SF's were compared with two control groups. SF sufferers were significantly lighter, smoked less and reported less of previous stress fractures as compared with controls. The levels of osteocalcin and alkaline phosphtase were significantly higher in SF patients and vitamin D lower. For the genetic workup, we have optimized the PCR and DGGE systems for almost all published exons of the procollagen genes, and have detected 5 polymorphism's and one potential splice junction mutation and a mutation in a patient with Oseogenesis Imperfecta. In addition, we have analyzed a subset of soldiers for allelic patterns of the vitamin D and calcium sensing receptors. This serves as proof for the ability of the system to detect sequence alterations, and that they do occur in a subset of soldiers. In addition, the potential to use the biochemical analyses as aid in diagnosis of SF needs to be further explored.

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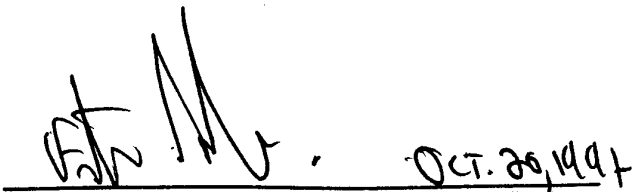

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Introduction

General background

Several lines of indirect evidence made it plausible (and testable) that in a subset of soldiers with stress fractures (SF) there may be a genetic component (Friedl et al 1992; Giladi et al 1986; Milgrom et al 1985; Singer et al 1990). Specifically, predisposing mutation(s) in one of the two genes encoding for procollagen type 1 (*COL1A1 & COL1A2*) (Procop 1992). These genes are the underlying cause of Osetogenesis Imprefecta (OI), both severe and mild forms (Spotila et al 1994). We hypothesized that subtle mutations within these two genes, that under normal circumstances would not result in any phenotype, may result in stress fractures given the special workload placed on young training soldiers.

Recently, several publications further support the notion that there may be a genetic component to stress fractures: the correlation between bone mass, a primarily genetically determined parameter (Pocock et al 1987; Krall et al 1993; Soroko et al 1994) and the risk for stress fractures in US soldiers (Kimmel et al 1996; Armstrong et al 1996), the correlation between bone mass and the polymorphism in the Sp1 binding site in the promotor region of the procolagen type ! gene (Grant et al 1996), and the occurrence of multiple stress fractures in US women during basic training (Lappe et al 1996).

Several other genes may also be considered as candidate genes to be involved in the genetic predisposition to SF, primarily those that are involved in pathological bone conditions and osteoporosis: vitamin D receptor, estrogen receptor and calcium sensing receptor. Specific allelic patterns within these genes or even mutations, have been identified in patients with a variety of bone pathologies (Husmeyr et al, 1994, Morrison et al, 1994 Sano et al, 1995 Smith et al, 1994)

Study design

To test this hypothesis, we have identified soldiers with clear-cut evidence of stress fractures (clinically and by bone scan), gathering all the pertinent data from these individuals (clinical, epidemiological, biochemical analysis of bone turnover parameters, and bone scan data). Constitutional DNA is extracted from peripheral blood leukocytes, and analyzed for the existence of mutations within the two mentioned genes and the other candidate genes by PCR amplifications and denaturing gradient gel electrophoresis (DGGE) (Myers et al 1987), complemented by DNA sequencing (Syvanen et al 1989) of amplified PCR products that display abnormal migration pattern. In addition, for the genes that have polymorphisms within them (e.g., vitamin D receptor), we test for specific allelic patterns by restriction enzyme digest of the relevant PCR products. As controls we use two groups of soldiers: a symptomatic control group that is composed of age-matched controls with no objective evidence of stress fractures, and unit

and ethnically matched controls at the final stages of their basic training who are totally asymptomatic. The latter group only fills the detailed questionnaire, but undergoes no bone scan or DNA testing.

Body

Overall, 2591 soldiers participated in the study: 2306 (89%) were males and 285 (11%) were females. Several subgroups were defined: 304 soldiers (293 males and 11 females) were diagnosed with high grade (grade 3-4 by bone scan) stress fractures or more than 3 sites of grade 2 fractures (= SF group). One control group (symptomatic controls) included 237 soldiers (223 males and 14 females) with clinical symptoms compatible with stress fractures, but normal bone scan. A second control group was composed of 2050 asymptomatic soldiers (1790 males and 260 females) from the same units as the stress fracture patients, who filled the questionnaire during the last week of basic training. Several parameters failed to reach statistical significance in a uni- and multivariate analyses between the different groups. These included: ethnic origin, rural or urban residence, type of military service (combat or non-combat units), education levels, hand dominance, dietary calcium intake, and pre-recruitment physical conditioning regimes. Interestingly, none of the 13 soldiers who were observant Jews (of 1725 who answered the question) developed stress fracture. Characteristics that were statistically different in the SF group as compared with both control groups are summarized in table 1. Notably, male SF fracture patients were lighter than both controls, smoked **less**, had **less** of a family history of bone diseases and siblings with SF, and reported **less frequently** of previous stress fractures. The amount of smoking was inversely correlated with the risk for SF. Additionally, females with SF had a lower BMI and SF was diagnosed more often during basic training.

Biochemical analysis

Comparison of serum levels of 5 parameters associated with bone remodeling and collagen breakdown was carried out in 40 individuals in each of the SF group and an ethnic and unit matched symptomatic controls. The results are summarized in table 2. As is evident, bone specific alkaline phosphatase and osteocalcin levels were significantly higher in the SF group as compared with the controls. Surprisingly, vitamin D levels were significantly lower in the SF group. Moreover, bone specific AP levels of over 45 u/ml and osteocalcin over 12.5 ng/ml were only observed in the SF group.

All these data are being summarized and analyzed for an upcoming publication and were presented in a poster form at the recent ASBMR meeting (September 1997).

DNA analysis

Preliminary phase

For DGGE analysis, 59 of the 61 successfully amplified fragments have now been focused on DGGE. This analysis revealed five abnormal migration patterns: in exons 8 and in fragments containing exons 12-13 and exons 14-15 of the *COL1A1* and in exons 6 and 28 of the *COL1A2* . Sequence analysis of these PCR fragments, revealed an intronic polymorphism in intorns 12, and 14 and in exons 6 and 28, the known polymorphisms were confirmed. These finding is important for several reasons: first and foremost, it clearly demonstrates the ability of the DGGE to detect sequence alterations within these two genes. Second, it adds a tool to family analysis when trying to decide which of the two gene to analyze (*COL1A1* or *COL1A2*). Furthermore, it may provide a tool to demonstrate allelic disequilibrium between the tested (i.e., stress fracture patients) and the controls.

Laboratory analysis of DNA from soldiers

Three hundred and eighty nine individuals gave their informed consent and blood was withdrawn from them for DNA extraction. DNA has been extracted from 200 soldiers. As the original protocol indicates, there is no data in the laboratory to identify which sample belongs to the tested group and which one is of the control group. These data are being kept in a central computer at the site where the soldiers are being evaluated. The samples in the lab are known only by given numbers.

PCR amplification and DGGE analysis of 11 exons of the *COL1A1* (exons 3, 4, 6, 8, 11, 12, 13, 14, 15, 16, 50) and 7 of the *COL1A2* (exons 6, 10, 11, 12, 27, 29, 33) was done on all 200 samples. In the course of these analyses, abnormal migration pattern was detected in 6 samples in the exon 12 of the Col1A2 gene and found to be a base change which could lead to an alternative splicing. Moreover, a mutation within a glycine residue was detected in a single patients with OI (see attached abstract of the manuscript in press in Human Genetics). In addition, the setup for determining allelic patterns of the vitamin D receptor and the Calcium sensing receptor have all been set up in the lab, and 160 soldiers underwent the vitamin D receptor analysis and 80, calcium sensing receptor allelic analysis. Furthermore, we have identified a novel missense mutation within the estrogen receptor in a subset of individuals with osteoporosis, and we plan to look for that missense mutation in all soldiers.

Conclusions

The initial data is encouraging for several reasons. First and foremost, we were able to recruit a large cohort of soldiers and controls. Second, the mutational analysis scheme devised

has proven capable of detecting sequence alterations, including novel polymorphisms and potentially splice junction mutations. Third, allelic pattern determination of additional candidate genes has been set up and has been applied to the tested group of soldiers. The hypothesis that there may be a genetic component to stress fractures has been given another boost, by the fact that so many soldiers with stress fractures report of a family history of either stress fractures or "bone diseases". Unfortunately, we were unable to retrospectively ascertain these individuals, and their exact family history. Lastly, initial indication that biochemical analysis of soldiers with SF may be helpful in the diagnosis of SF. It is clear from these initial studies that more soldiers need to be analyzed, a larger control group needs to be recruited to assess the apparent protective effect of smoking, and that more genes need to be analyzed.

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TABLE 1 STATISTICALLY SIGNIFICANT CORRELATES OF STRESS FRACTURES

Parameter	P value univariate	P value multivariate
Stage of military training (females only)*	<0.0001	0.003
No previous stress fractures (males only)	0.001	0.003
No history of smoking (males only)	0.013	0.0132
Smoking intensity** (males only)	0.0001	0.0522
No stress fractures in sibs (males only)	0.02	
No family history of bone disorders (males only)	0.001	0.003
Lower weight (males only)	0.018	0.0092
Lower BMI (females only)	0.075	***

* More stress fractures during the basic training than at later stages of the military service

** The number of cigarettes consumed per day was higher in individuals with no stress fractures

*** Insufficient numbers to perform analysis

Table 2: LABORATORY EVALUATION OF BONE REMODELLING PARAMETERS

Marker	Controls mean (SD)	Stress fractures mean (SD)	P value
PTH pg/ml	25.4 (14.7)	28.7 (16.5)	NS
ICTP ng/ml	4.6 (2)	5.2 (1.6)	NS
25OHDng/ml	29.8 (8.5)	25.3 (10)	0.033
Osteocalcin ng/ml	8.8 (1.6)	10.8 (2.4)	0.00003
Bone AP U/L	26.2 (7.7)	37.6 (15.5)	0.0001

human genetics

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Professor Eitan Friedman
Oncogenetics Unit
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A missense mutation in Col1A1 in a Jewish Israeli patient with mild Osteogenesis Imperfecta, detected by DGGE.

Galia Gat-Yablonski*, Liat Ries*, Dorit Lev, Boleslaw Goldman* and Eitan Friedman***.**

Oncogenetics Unit , Institute of Genetics, Chaim Sheba Medical Center, Tel-Hashomer 52621, Israel. Fax: 972-3-535-7308; E-mail: feitan@post.tau.ac.il

*The first two authors have equally contributed to the work presented in this paper.

**Present address: Institute of Genetics, Wolfson Medical Center, Holon, Israel.

***To whom correspondence should be addressed.

Abstract

Osteogenesis Imperfecta underlie germline mutations in either *Col1A1* or *Col1A2*. Here we describe, for the first time, the use of the DGGE technique for mutation analysis of the *Col1A1* gene. By employing this technique, we identified a point mutation in a young Jewish-Israeli patient with mild OI. The missense mutation, a G to A alteration at position 888, result in a Gly to Arg substitution at codon 79. Furthermore, the patient's mother who was clinically labeled as OI, solely based on the fact that she has blue sclera, was found not to carry this mutation in two different tissues. We suggest that blue sclera alone should not be used as a parameter for the diagnosis of OI, and that DGGE can be effectively used for mutation analysis of the *Col1A1* gene.